AD-A179 160 MASS-SCREENING OF CURARIMIMETIC NEUROTOXIN ANTAGONISTS 1/1
(U) STATE UNIV OF NEW YORK AT STONY BROOK DEPT OF
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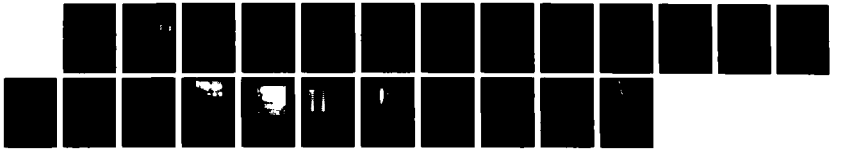

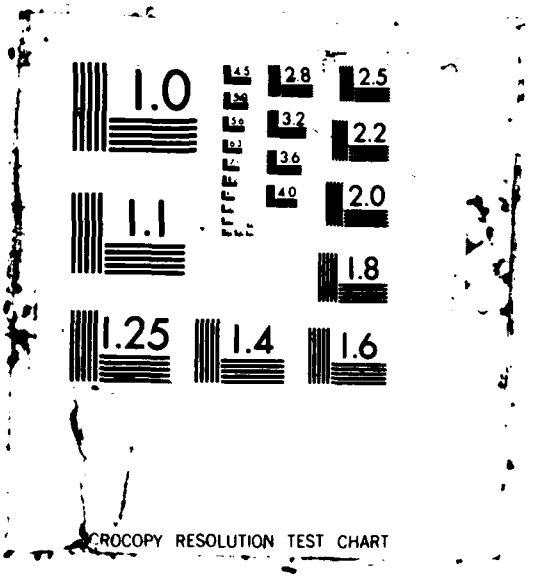
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MASS-SCREENING OF CURARIMIMETIC NEUROTOXIN ANTAGONISTS

ANNUAL REPORT

JAKOB SCHMIDT

FEBRUARY 15, 1987

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6058

Department of Biochemistry
State University of New York at Stony Brook
Stony Brook, L.I., New York 11794

SECURITY CLASSIFICATION OF THIS PAGE

AD-A179160

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Department of Biochemistry State University of New York 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) at Stony Brook Stony Brook, L.I., New York 11794	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable) SGRD-RMI-S	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-86-C-6058
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1-622[illegible]71; TASK NO. AA; WORK UNIT ACCESSION NO. 379

11. TITLE (Include Security Classification)
(U) Mass-Screening of Curarimimetic Neurotoxin Antagonists

12. PERSONAL AUTHOR(S)
Schmidt, Jakob

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15 PAGE COUNT
Annual	FROM 2/1/86 TO 1/31/87	1987 February 15	20

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Curarimimetic - neurotoxin - acetylcholine receptor - neuromuscular junction - antidote - mass screening
06	01		
06	15		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The present search for antidotes of curarimimetic neurotoxins is based on the premise that these toxins are considerably larger than the neurotransmitter acetylcholine, and that therefore the toxin binding site on the receptor must comprise elements other than the acetylcholine site itself. Consequently toxin antagonists can be envisaged which prevent toxin binding and action, without being themselves activators or inhibitors of the receptors. We have obtained evidence that several nontoxic small basic proteins function as such toxin antidotes in vitro. To aid in the mass screening of other antidote candidates we have shown that the DEAE cellulose disk technique, a standard receptor assay, can be adapted for large-scale screening. Preliminary results also indicate that Elisa versions of the mass screening method are feasible.

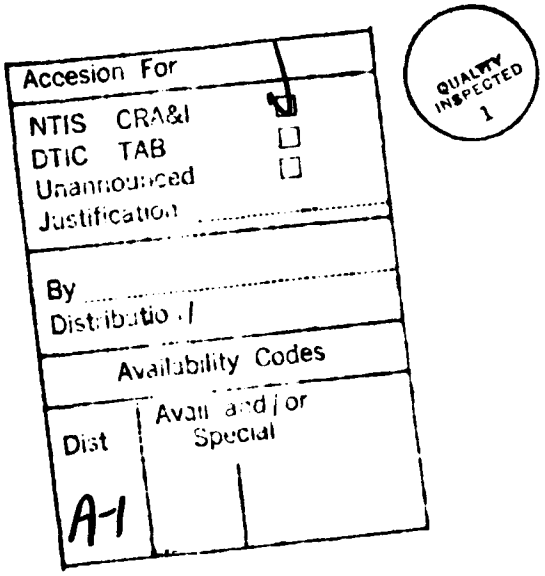
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c OFFICE SYMBOL
Mary Frances Bostian	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 Previous editions are obsolete SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).



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STATEMENT OF PROBLEM

The objective of this research is to develop methods which will eventually lead to the identification of useful antagonists of curarimimetic neurotoxins, a group of snake venom proteins which bind to and inactivate the nicotinic acetylcholine receptor in the neuromuscular junction. The great majority of neurotoxin antagonists, i.e. substances that in vitro compete with or displace the neurotoxin, are likely to be receptor agonists or antagonists themselves, and therefore of little therapeutic value. It is therefore anticipated that the search for the relatively rare physiologically inert toxin antagonists will require a major effort which could be greatly helped by the development of labor-saving mass-screening methodologies.

BACKGROUND

Identification of ligands by radioligand competition has been practiced widely in the field of neurotransmitter receptor research, e.g. to establish the "pharmacological profile" of a radioligand binding site and to correlate such binding data with clinically observed drug potencies (Creese, 1985). In studies of cholinergic receptors in the CNS, inhibition of acetylcholine binding to brain membranes by appropriate ligands has been used to identify a binding site as either nicotinic or muscarinic (Schwartz et al., 1982). Radioreceptor assays have been used to quantitate known receptor specific drugs (Enna, 1985) as well as to identify and develop novel compounds with desired receptor binding properties (Snyder, 1983; Evans et al., 1986). This approach has not been used in the search for acetylcholine receptor toxin antidotes, although data describing the potency of various drugs in neurotoxin competition assays are scattered throughout the literature (see e.g. Weber and Changeux, 1974; Schmidt and Raftery, 1975; Neubig and Cohen, 1979; Blanchard et al., 1979). In all of these studies the goal was to obtain information on the nicotinic nature of the binding site; or to quantitatively determine ligand affinities; or to study the phenomenon of desensitization. Consequently analysis of a relatively small number of ligands sufficed, and it was possible to employ various toxin binding assays (based on gel filtration, ion exchange chromatography, even sucrose density gradient centrifugation and immunoprecipitation), without regard to their speed and simplicity.

RATIONALE

The assumption on which this research project rests is that the neurotoxin binding site in the nicotinic acetylcholine receptor

differs sufficiently from the acetylcholine binding site that drugs can be found or designe' which discriminate between them. In particular, the expectation is that compounds will be found that interact with portions of the toxin binding site without interfering with the binding of the agonist, and therefore act as physiologically inert toxin antagonists. In order to corroborate and solidify this working hypothesis we have conducted drug binding studies using conventional techniques. The results suggest that compounds that preferentially compete for the toxin site can indeed be found. Further studies of this sort depend on the design of mass screening procedures which we have begun to develop on the following premises: (1) Acetylcholine receptors are highly conserved proteins; it therefore suffices to establish the inhibitory potency of a candidate antagonist on the interaction of alpha-bungarotoxin and Torpedo californica receptor to predict the effectiveness of the potential antidote with respect to most other receptors, including those from human muscle. (2) It should be possible to combine simple toxin binding procedures currently in use, with mass-screening procedures developed for hybridoma work.

METHODS AND MATERIALS

(1) Ligand preparation:

^{125}I-alpha-bungarotoxin: Radioiodinated alpha-bungarotoxin was prepared largely as described in the original proposal. Briefly, the native toxin was prepared from B. multicinctus venom (Miami Serpentarium, Miami, FL) by chromatography on carboxymethyl-cellulose (Whatman CM 52) and Sephadex G-50 (Pharmacia, Piscataway, NJ), and iodinated by means of the chloramine T procedure as described by Wang and Schmidt (1980). During the course of the year three batches of radiotoxin were produced, each lasting for 2 to 3 half-lives (60 days). Specific activities ranged from ca 1000 cpm/femtomole to 150 cpm/femtomole, depending on batch and age, for the monoiodinated derivative, and about twice as high for the diiodinated preparation.

[^{3}H]-Acetylcholine: Tritiated acetylcholine was synthesized according to Schwartz et al. (1982), by acetylation of [methyl-^{3}H]choline chloride (78 Ci/mmol; Amersham Corp.).

(2) Receptor preparation

(a) Crude membranes. T. californica acetylcholine receptor was prepared essentially as described in the proposal. Briefly, frozen electric tissue (obtained from Marinus Corp., Long Beach, CA) was thawed in 10 mM sodium phosphate, 10 mM sodium azide, 5 mM sodium EDTA, 5 mMEGTA, 10 mM iodoacetic acid, 1 mM phenylmethylsulfonyl fluoride and homogenized, and the homogenate subjected to low-speed centrifugation. Receptor-enriched membranes were obtained by ultracentrifugation of the resulting supernatant.

(b) Partly purified membranes. The crude membrane pellet was resuspended, layered on top of a 5 to 20% sucrose gradient, and centrifuged as described by Duguid and Raftery (1974). In some cases, to accomplish further purification, such membranes were extracted at low ionic strength and high pH, as described by Neubig et al. (1979).

(c) Solubilized receptor. Partly purified membranes were dissolved in 1% Triton X-100, followed by recentrifugation at high speed to remove detergent-insoluble matter.

(d) Purified receptor. The detergent extract was subjected to affinity chromatography on immobilized Naja naja siamensis alpha-toxin, as described by Lindstrom et al. (1982). All types of receptor preparation (membrane-bound, solubilized, and purified), when stored at 4° in 5 mM sodium azide, were stable for periods of several months.

(e) Other receptor sources. As Torpedo and Homo represent extreme poles of vertebrate evolution, an attempt was made to replace Torpedo electric tissue with chick skeletal muscle, since other research in the lab deals with the avian acetylcholine receptor. This approach was given up because of the disproportionate effort for the production of relatively small quantities of receptor and because of the difficulties encountered in purifying the chick muscle receptor.

(f) Subunit isolation. One batch of purified Torpedoreceptor was subjected to SDS polyacrylamide gel electrophoresis, using the protocol of Laemmli (1970). Care was taken not to boil the sample, as this leads to smearing of the alpha band. After electrophoresis the individual bands were located by staining of a gel strip with Coommassie Blue and the gel slice corresponding to the alpha band excised and electroeluted.

(3) DEAE-cellulose Disk Assay

Binding of radioiodinated alpha-bungarotoxin to solubilized Torpedo receptor was measured using the DEAE-cellulose disk assay as originally described by Schmidt and Raftery (1973). Receptor quantitation was usually carried out in quadruplicate. Increasing volumes of the receptor-containing sample are brought to 0.1 ml with 10 mM sodium phosphate, 0.1% Triton X-100, and 0.025 ml of radiotoxin added are such that some or all incubations are at toxin excess. After 30 min at room temperature 0.1-ml aliquots are pipetted onto 25 mm-diameter disks of DEAE-cellulose paper (Whatman DE 81), and the loaded disks immersed in a beaker containing 10 mM sodium phosphate, pH 7.4, 0.1% Tritin X-100. After three changes of wash fluid over a period of 30 min, the disks are removed, air dried, and counted in a gamma spectrometer. Nonspecific binding was defined as binding observed in the presence of excess nonradioactive toxin; for samples of high receptor content (as is usually the case with Torpedo preparations) it suffices to determine binding of radiotoxin to DEAE-cellulose in the absence of receptor (see Fig. 3).

(4) Preparation of anti-receptor antibodies

Antisera: For production of polyclonal antibodies , purified receptor (or receptor subunit) was emulsified with complete Freund's adjuvant and administered in small quantities (10 to 100 ug) intradermally into rabbits. Booster injections were given in 2-week intervals. Blood samples were obtained through the ear vain, for both analytical and preparative purposes. Titers were determined by a double immunoprecipitation technique, using complexes of Torpedo receptor and ^{125}I-alpha-bungarotoxin as antigen, and a goat anti-rabbit immunoglobulin antiserum as secondary antibody; titers are expressed in moles of toxin binding sites precipitated per 1 of serum. Three antisera were produced against purified receptor, with titers of 6.0, 2.5, and 3.0 uM; three antisera were obtained against the isolated alpha subunit (anti-receptor titers: 0.7, 0.8, and 0.4 uM). An antiserum against alpha-bungarotoxin was also generated for eventual use in ELISA assays (titer: 5×10^{-6} M, measured by double immunoprecipitation of radioactive toxin).

Monospecific antibodies: Receptor-specific antibodies were isolated by affinity chromatography on immobilized T. californica acetylcholine receptor. The affinity resin was prepared as follows: sepharose 4B-CL (Pharmacia) was activated by the CNBr procedure, and reacted with alpha-bungarotoxin; this toxin resin was incubated with a Triton X-100 extract of electric tissue, washed, and treated with dimethylpimelimidate as described by Schneider et al. (1982), to achieve covalent coupling of the receptor to the resin. Specific antibodies were then adsorbed onto this resin out of an anti-receptor antiserum, eluted from the washed resin with 4 M guanidinium isothiocyanate, dialyzed, and lyophilized. Monospecific anti-alpha-bungarotoxin antibodies were similarly obtained by affinity chromatography on resin-coupled antigen.

Monoclonal antibodies: The hybridoma TIB 175 was obtained from the American Type Culture Collection (Rockville, MD). It produces mAb 35, a monoclonal antibody directed against the alpha-subunit of the acetylcholine receptor from a wide variety of species (Tzartos et al., 1981; 1982). Cultures were grown according to the recommendations of the supplier. The supernatant was subsequently concentrated by ultrafiltration through an Amicon XM 50 filter, and immunoglobulins precipitated with ammonium sulfate (33% saturation) and further purified by chromatography on DEAE-cellulose, desalted, and lyophilized.

Secondary antibodies: Goat anti-rabbit immunoglobulin antiserum was prepared as described in Campbell et al. (1970); goat anti-mouse immunoglobulin antiserum was obtained from Pel-Freez (Rogers, AR).

(5) Drug competition experiments:

Nicotinic drugs (and other agents) were serially diluted into low-ionic strength buffer (10 mM sodium phosphate, pH 7.4; 10 mM sodium azide; 50 um eserine; low ionic strength is required to

increase toxin binding rate). Alkaline-extracted electric organ membranes were added to a final receptor concentration of 3.1 nM and incubated for 60 min at room temperature. The suspensions were then divided and combined with either [^{3}H]-acetylcholine (final concentration 9 nM) or ^{125}I-alpha-bungarotoxin (final concentration 1 nM). The acetylcholine-containing samples were incubated for 40 min at 4^{o} and filtered through Whatman GF/C glass fiber disks, and the disks washed with three 5-ml volumes of ice-cold buffer. The radiotoxin-containing samples were incubated at room temperature for 20 min (while toxin binding proceeds approximately linearly) and assayed for bound toxin by the DEAE-cellulose disk technique. Inhibition constants (K_I) were calculated using the expression $K_I = IC_{50}/(1 + ([[^3H]AcCh]/K_D)$, where IC_{50} is the midpoint of the inhibition curve; the K_D of acetylcholine binding to Torpedo membranes is 4 nM. The protection constant (K_P) is the drug concentration at which the initial rate of toxin binding is reduced to half. Data on the effect of l-nicotine are presented as an example in Fig. 2.

RESULTS AND DISCUSSION

1. A comparison of toxin and acetylcholine binding sites in the acetylcholine receptor from the electric tissue of Torpedo californica

In contrast to many small and water-soluble proteins which are amenable to detailed structural analysis by x-ray crystallography, the structure of functionally significant sites on large membrane proteins remains largely unknown. This is a consequence of their size, insolubility, and of the microheterogeneity in their carbohydrate moieties. A case in point is the nicotinic acetylcholine receptor from skeletal muscle. Although the primary structure of all subunits of this receptor from a number of species has been completely elucidated, and although, furthermore, the quaternary structure has been worked out to a considerable extent, elucidation of the precise geometry of the acetylcholine binding site through which nicotinic agonists activate the receptor is a long way off. Thus the design of ligands for this site is by necessity an empirical rather than a rational one.

This also holds for the very important question whether inhibitors of curarimimetic toxins can be designed in such a way as not to interfere with the normal activity of the acetylcholine receptor in the neuromuscular junction. Although there is some indirect evidence that inorganic cations weaken the interaction of the receptor with alpha-bungarotoxin, without equally affecting the function of the receptor (see Schmidt and Raftery, 1974), I decided to perform some preliminary experiments on whether compounds can be found that protect against the blocking action of alpha-bungarotoxin without themselves being receptor agonists or antagonists. This is a question that according to the original schedule was supposed to be addressed

in the third year of the contract, but which I felt I had to answer in the affirmative, at least tentatively, before embarking on purely methodological aspects.

That the binding sites for acetylcholine and alpha-bungarotoxin should not be identical is a plausible postulate based on a comparison of the size, structure, and affinity of these 2 ligands. While the agonist is a small molecule (MW for the free base: 146 daltons) with a binding energy of about 7 to 11 kcal/mol, the toxin has a MW of 8,000 daltons and binds virtually irreversibly, with an association energy in excess of 20 kcal/mol, implying a larger number of interactions with the receptor molecule.

To obtain additional experimental evidence, we carried out thermal inactivation studies. The melting temperature for the acetylcholine binding site is somewhat lower than that for the toxin site (see Fig. 1), in support of the notion that, although the sites overlap, a partial unfolding, sufficient for the loss of agonist binding, leads only to partial disruption of the larger toxin binding site; in other words, the acetylcholine site represents a subsite of the toxin site.

More recently we have turned to a comparison of the drug affinities of the 2 binding sites as a more convincing and also more relevant study. Again the assumption is that the toxin binding site encompasses the acetylcholine site but extends beyond it and that it should therefore be possible to identify compounds or groups of compounds with the desired discriminatory properties. Using a filtration assay that can likewise be used for toxin and acetylcholine binding, we screened a number of cholinergic drugs as well as various other compounds for their ability to inhibit the association of the radioactive ligands. Some of the results are shown in Table I. An inspection of the effect of cholinergic drugs reveals excellent correlation of the two pharmacologies suggesting that the drugs attach to the same site to bring about inhibition of acetylcholine and toxin binding, but this correlation breaks down when nonspecific cationic substances are tested. The parameter recorded in the fourth column (K_I/K_P) measures the preference of a substance for the toxin binding site; it appears that small basic proteins are capable of blocking toxin binding over a wide range of concentrations without any detectable effect on acetylcholine binding.

Taken together these findings suggest to us that the toxin site is larger than the agonist site, as could have been reasonably expected, and that it comprises additional negative subsites, for which ligands might be designed that will not interfere with receptor activation. It is conceivable that the design of toxin antagonists will be based on modifications of small basic proteins, perhaps even on fragments or on chemical derivatives of the toxins themselves.

2. An autoradiographic version of the DEAE-cellulose technique of the toxin binding assay

The standard DEAE-cellulose assay and its principle: The DEAE-cellulose adsorption assay which has been a mainstay of receptor quantitation over the past 15 years exploits the difference in isoelectric point between free and receptor-bound ^{125}I-alpha-bungarotoxin (ca 9 and 5, respectively). Briefly, the assay involves pipetting the sample (which previously has been incubated with an excess of radiotoxin) onto a disk of DEAE-cellulose paper to which the receptor-toxin complex binds strongly at low ionic strength; washing off unbound radiotoxin; and determining disk-bound radioactivity in a gamma counter (Schmidt and Raftery, 1973; for technical detail see Methods).

An autoradiographic version of the DEAE-cellulose assay: Instead of counting bound radiotoxin in the gamma spectrometer, it can be detected by autoradiography. This is shown in Fig. 3, where data obtained by the standard assay and autoradiographic analysis of the disks used in the standard assay are compared. Fig. 4 shows the use of a single sheet of DEAE-cellulose for the autoradiographic quantitation of multiple receptor samples; clearly sample volumes and spacings can be reduced to fit in the dimension of multiwell titer plates. Fig. 5 demonstrates application of the DEAE-cellulose sheet version to a toxin-ligand inhibition study. It is clear from an inspection of the autoradiograph that the test compound, d-tubocurarine, competes for the toxin binding site, but it is less obvious how high its affinity for the site actually is. To determine this, the autoradiograph has to be quantitated. Such quantitation is usually accomplished by densitometry which in turn requires that the sample spots be of a particular shape. We have used a "slot-blot" device to generate linear rather than concentric spots which can subsequently scanned by a densitometer. The densitometric trace shown in the inset to Fig. 5 can be analyzed like an inhibition curve generated from cpm data to produce the desired K_D value (approximately 0.2 uM, in agreement with results obtained in the standard assay procedure).

(3) Preliminary Work on Solid-Phase Assays

Solid-phase assays offer certain advantages. Since toxin quantitation is performed directly in the container which is used for the incubation, a sample transfer step is eliminated; more importantly solid-phase assays lend themselves to modifications that would obviate the use of radioisotopes.

A prerequisite for solid-phase assays is the immobilization of the acetylcholine receptor onto the wall of the container, i.e. the individual wells of a microtiter plate. Since detergent-solubilized receptor sticks poorly to plastic, we have compared several methods of anchoring it. Results of such an experiment are shown in Fig. 7. As can be seen, there is only one efficient anchoring agent, namely affinity-purified anti receptor antibody. Antisera against the receptor are hardly better than no anchoring agent at all, presumably because of competition of nonspecific serum proteins for binding sites

on the plastic. Disappointingly, the monoclonal antibody does not seem to work very well either, possibly because of poor adsorption to the plastic. Concanavalin A, on the other hand, may not work because of the competition of a large number of glycoproteins in the crude extract for lectin sites. Results of experiments in which intact, receptor-containing membranes were used instead of extracts revealed that intact membranes adhere poorly to plastic, and that adhesion is not much improved even when wells are coated with purified receptor-specific antibodies (data not shown). Since preparation of monospecific receptor antibodies is costly, it will be important to examine other ways of receptor attachment. Reduction of detergent concentration or use of detergents other than Triton X-100 should be tested.

While solid-phase assays can be used in conjunction with radioligands as described above, they also lend themselves to nonradioactive versions. An increased number of manipulations is the price one has to pay for the elimination of radioisotopes. Nevertheless, in combination with multiwell titer plate and ELISA techniques, solid-phase assays appear as promising mass screening procedures. As of the time of writing this work is in progress.

Table I. Drug Affinities for the Acetylcholine- and Alpha-bungarotoxin Binding Sites of the Torpedo californica Acetylcholine Receptor

Membrane-bound Torpedo receptor was incubated with the indicated radioligand and serial dilutions of the indicated drug, and inhibition curves obtained as described for l-nicotine in Fig. 1. The C_{50} values obtained in the [^{3}H]-acetylcholine series were converted to K_I values as described in the Methods section.

Drug	K_I (M) Acetylcholine	K_P (M) Alpha-bungarotoxin	K_I/K_P
Nicotinic drugs			
Acetylcholine	4.0×10^{-9} *	7.9×10^{-8}	0.051
Benzoquinonium	8.0×10^{-7}	7.9×10^{-6}	0.10
Butyrylcholine	9.0×10^{-10}	2.0×10^{-8}	0.045
Carbamoyl choline	1.3×10^{-7}	1.4×10^{-4}	0.93
Choline	3.1×10^{-4}	8.9×10^{-6}	0.35
Cytisine	3.0×10^{-7}	4.0×10^{-6}	0.075
Decamethonium	4.2×10^{-7}	1.3×10^{-6}	0.32
Gallamine	3.5×10^{-7}	1.8×10^{-6}	0.19
Hexamethonium	5.1×10^{-6}	3.2×10^{-5}	0.16
Lobeline	1.6×10^{-6}	4.7×10^{-6}	0.34
d-Nicotine	5.7×10^{-6}	4.5×10^{-5}	0.13
l-Nicotine	1.6×10^{-6}	6.0×10^{-6}	0.27
Suberyldicholine	4.5×10^{-10}	1.5×10^{-9}	0.30
Succinylcholine	3.2×10^{-8}	3.1×10^{-8}	1.03
d-Tubocurarine	1.3×10^{-7}	1.8×10^{-7}	0.72
Other compounds			
Arecoline	6.3×10^{-5}	1.4×10^{-4}	0.45
Atropine	6.3×10^{-5}	1.2×10^{-3}	0.053
Hemicholinium	1.8×10^{-5}	2.0×10^{-5}	0.90
Lidocaine	1.0×10^{-2}	3.1×10^{-2}	0.32
Mecholyl	3.5×10^{-4}	2.2×10^{-3}	0.16
Muscarine	5.6×10^{-4}	1.0×10^{-3}	0.56
Oxotremorine	6.3×10^{-6}	4.5×10^{-5}	0.14
Pilocarpine	1.6×10^{-3}	1.7×10^{-2}	0.094
Procaine	7.0×10^{-4}	8.9×10^{-3}	0.079
TEA	7.0×10^{-4}	1.1×10^{-2}	0.064
TMA	2.8×10^{-5}	6.3×10^{-5}	0.44
Arginine	1.6×10^{-1}	3.6×10^{-2}	4.44
Lysine	1.3×10^{-1}	4.4×10^{-7}	2.95
Protamine	1.6×10^{-7}	1.1×10^{-5}	1.45
Cytochrome c	10^{-2}	2.5×10^{-6}	400
Lysozyme	10^{-3}	1.2×10^{-6}	833

* K_D determined by radioligand binding

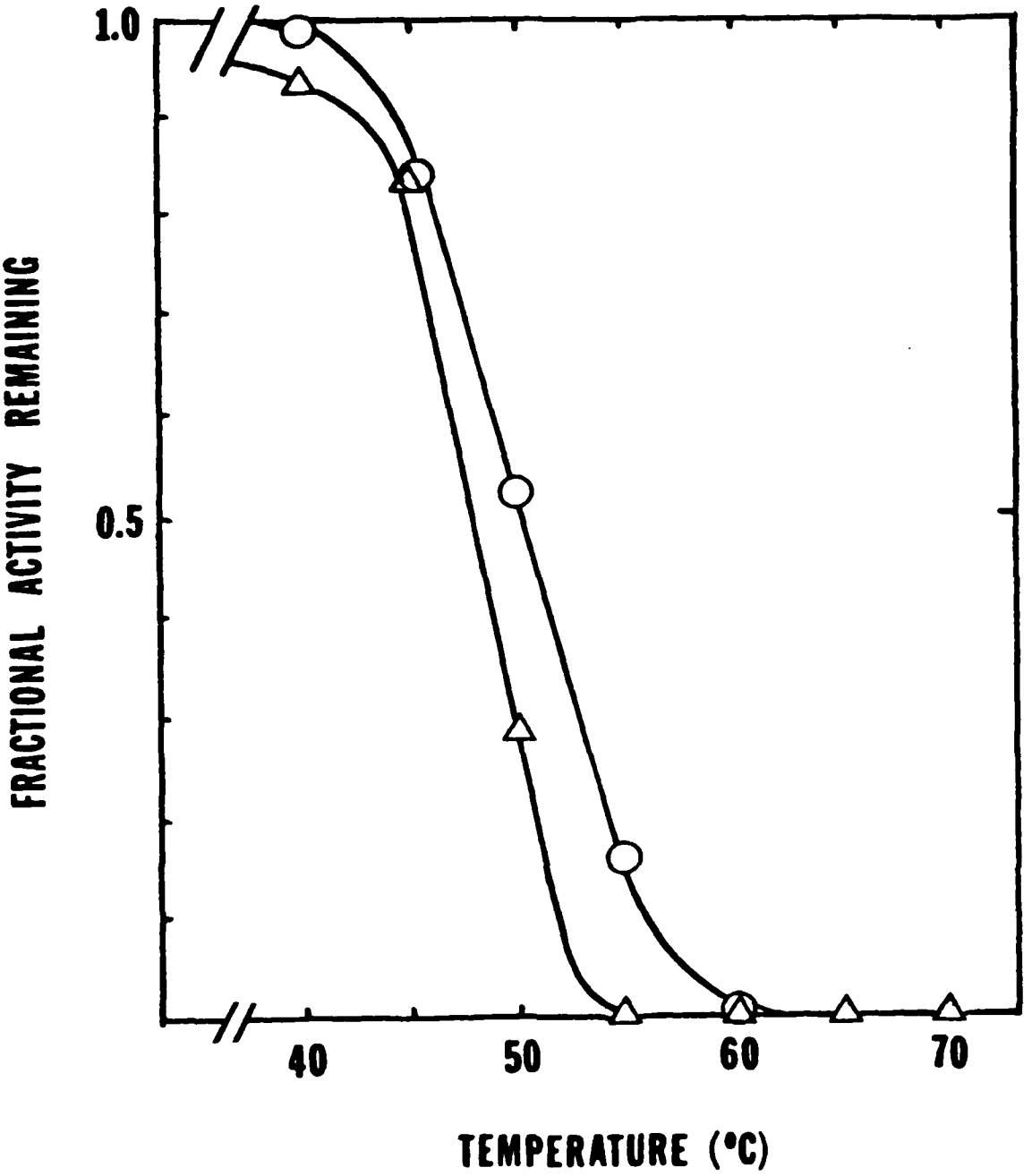

Fig. 1. Thermal Inactivation of Ligand Recognition by Torpedo californica Acetylcholine Receptor

Membranes were partly purified from a crude electric tissue particulate fraction as described in Methods and diluted with 10 mM sodium phosphate, pH 7.4 to a concentration of 2×10^{-9} M in binding sites. Aliquots were kept at the indicated temperature for 10 min, transferred to an ice bath, and assayed for [^{3}H]-acetylcholine (△) and ^{125}I-alpha-bungarotoxin (○) binding activity. Residual binding activity is presented normalized to a room temperature control.

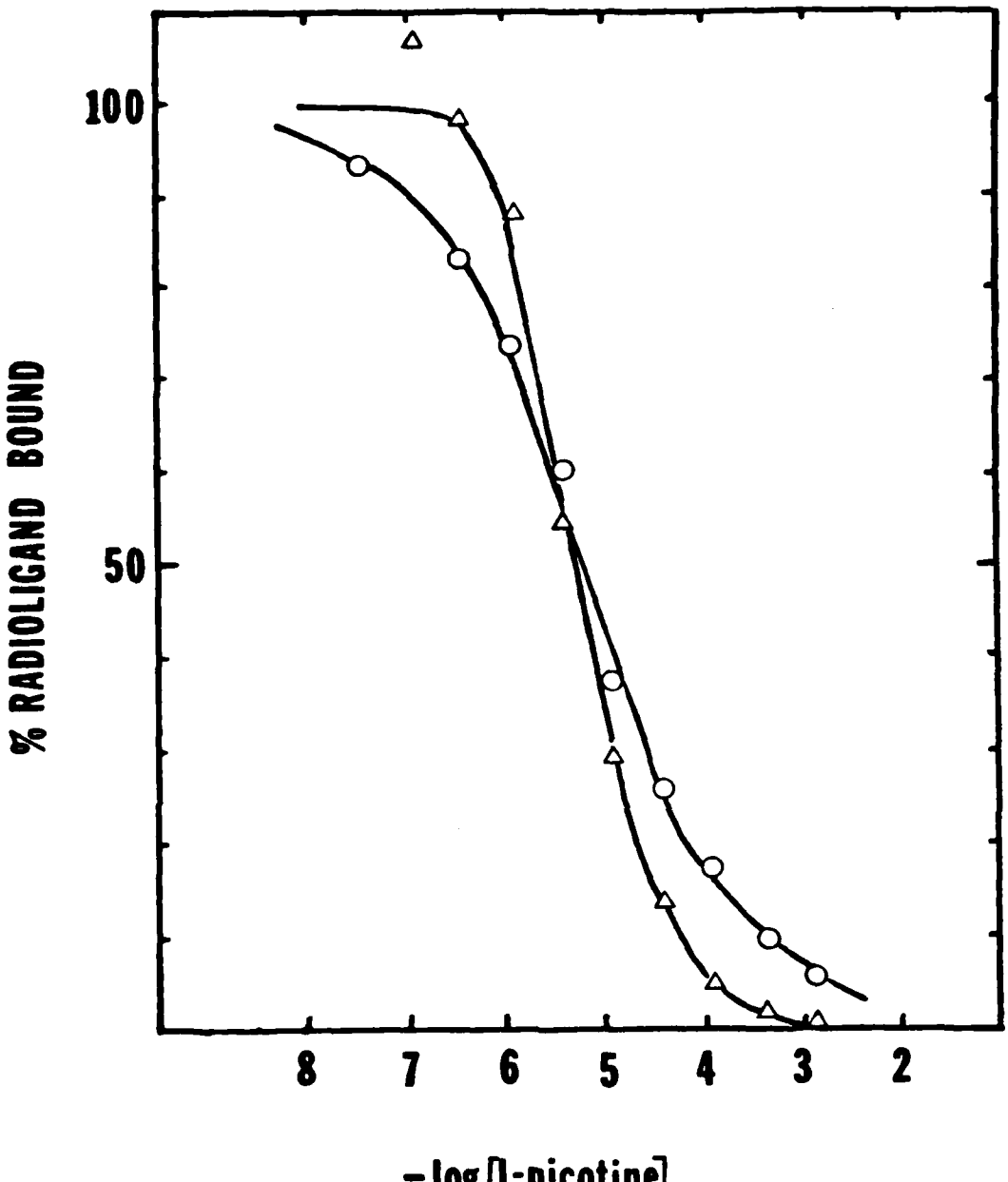

Fig. 2. Inhibition of Radioligand Binding to Torpedo californica Acetylcholine Receptor by l-Nicotine

Membrane-bound Torpedo receptor was tested in the presence of serially diluted l-nicotine for the binding of radioligand (^{125}I-alpha-bungarotoxin: ○; [^{3}H]-acetylcholine: △) as described in the Methods section. Binding data are normalized to a control without drug.

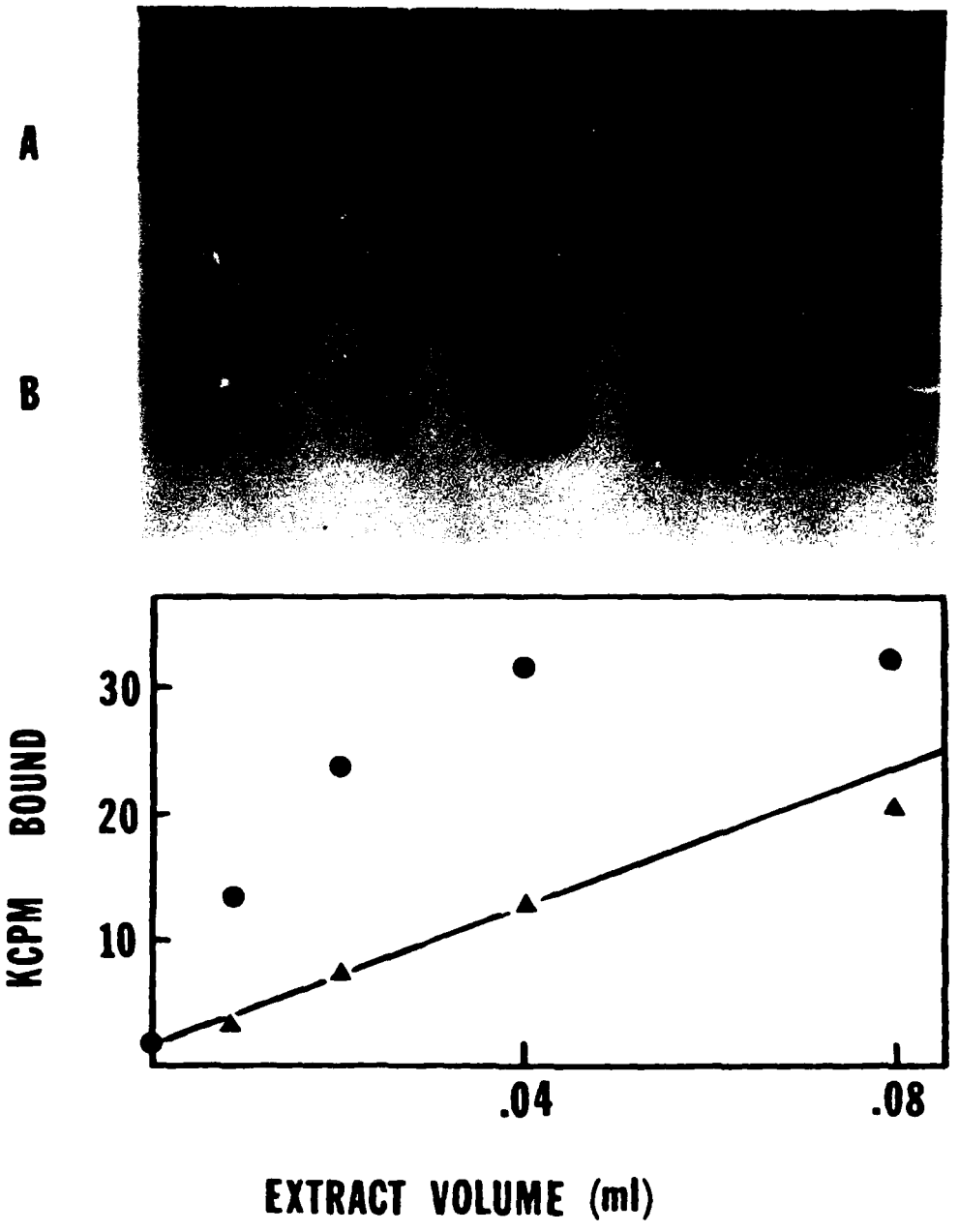

Fig. 3. Autoradiographic Representation of DEAE-cellulose Disk Assay

Torpedo electric tissue was extracted with Triton X-100. Increasing volumes of two dilutions, A (2,500ml/g original tissue; ●) and B (25,000 ml/g original tissue; ▲), were analyzed for binding of ^{125}I-alpha-bungarotoxin (at 5 x 10^{-10} M) by the standard DEAE-cellulose disk assay. Disk-bound radioactivity was quantitated in a gamma spectrometer at a counting efficiency of 80% (lower panel). The filter disks were then apposed to Kodak XAR-5 film; after exposure for 15 h at room temperature the film was developed (upper panel).

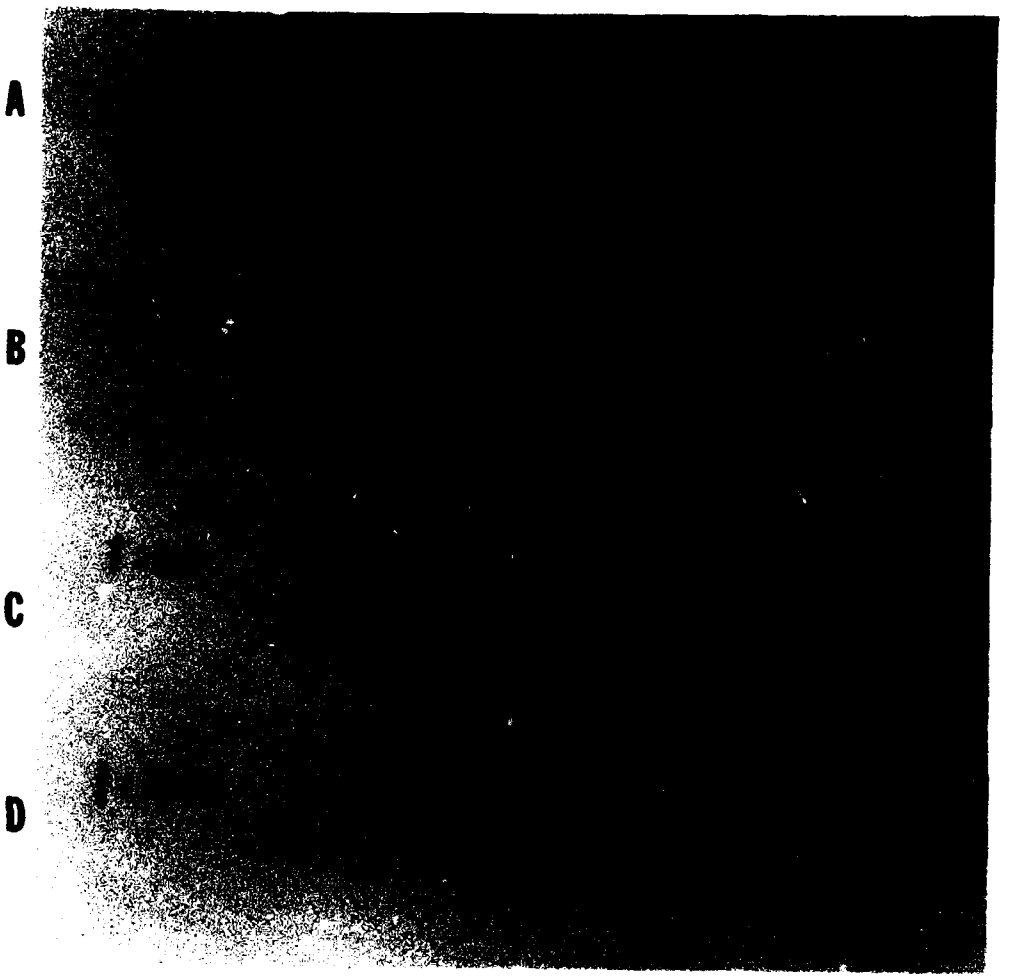

Fig. 4. Adaptation to DEAE-cellulose Paper Sheets

Increasing quantities of Torpedo electric tissue extract (final concentrations of 0; 0.64; 1.6; 2.7; 3.2; 4.8; 6.4; and 64 nM in toxin binding sites), diluted in "wash buffer" (10 mM sodium phosphate, pH 7.4, 0.1% Triton X-100) were incubated, in a total volume of 250 ul, with ^{125}I-alpha-bungarotoxin (final concentration 12 nM; total radioactivity 400,000 cpm). After 30 min at room temperature 100-ul aliquots were filtered, with mild suction, through a "dot blot" device (Bethesda Research Labs.) containing a sheet of DEAE-cellulose paper (row A). Rows B to D represent experiments in which 100-ul aliquots of 10-; 100-; and 1,000fold dilutions of the samples in row A were filtered, containing 16,000; 1,600; and 160 cpm of radioligand, respectively. After removal from the blotting device the sheet was placed in a shallow pan and rinsed with three changes of "wash buffer" over a period of 30 min. The washed sheet was air-dried, apposed to a pre-flashed film of Kodak XAR-5, and the film developed after an overnight exposure at room temperature, using an intensifying screen.

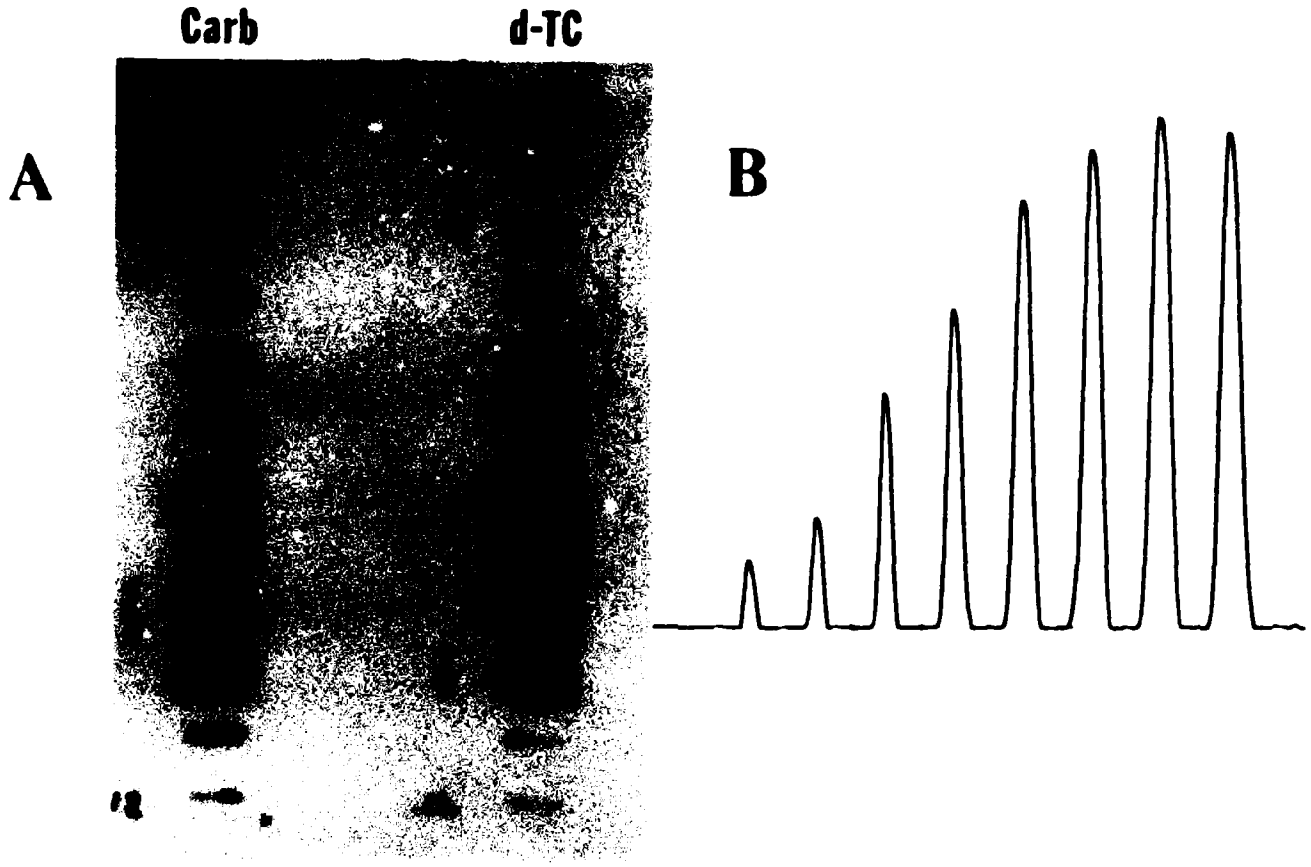

Fig. 5. A Quantitative Competition Experiment Using Autoradiography

A: Serial 1:2 dilutions of carbamoyl choline ("Carb") and d-tubocurarine ("d-TC") in "wash buffer" (see legend to Fig. 4) were prepared in multiwell titer plates, and then Torpedo extract and radiotoxin added to final concentrations of 0.6 and 6 nM, respectively. The highest final concentration of carbamoyl choline was 20_{6}mM, that of d-tubocurarine 20 uM. Controls included presence of 10^{-6} M nonradioactive toxin (slot 11) and absence of receptor (slot 12). After incubation at room temperature for 30 min, 100-ul aliquots were filtered through DEAE-cellulose clamped into a "slot blot" device (Minifold II, Schleicher & Schuell). The sheet was then washed, dried, and autoradiographed.

B: Densitometric trace of the curare experiment (slot 3 through 10).

Fig. 6. Anchoring Torpedo Acetylcholine Receptor to multiwell titer plates

Rows of wells in a multiwell titer plate (Falcon) were filled with, from left to right, A: no addition; B: a serum directed against a segment of the mouse muscle acetylcholine receptor alpha subunit; C: a serum against the alpha subunit of the Torpedo acetylcholine receptor; D: a monospecific antibody preparation against native Torpedo acetylcholine receptor; E: an anti alpha-bungarotoxin serum; F: an anti alpha cobrotoxin serum; G: concanavalin A; H: another anti alpha subunit serum; I: a serum directed against denatured Torpedo receptor; J: monoclonal antibody mAb 35; K: normal rabbit serum; and L: phosphate-buffered saline. After overnight treatment wells were washed and incubated with, consecutively, 1% BSA, Torpedo extract, and ^{125}I-alpha-bungarotoxin in the presence of a gradient of d-tubocurarine (bottom row 100 μM drug, serially diluted 1:5 in upper rows; top row drug-free). After washing out excess radioactivity, the plate was apposed to Kodak XAR-5 film, and autoradiography carried out as described.

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